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Studies of tobacco mosaic virus with fluorescent antibodies.

by G. Schramm and B. Roettger.

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A method for the study of plant tissue with the aid of fluorescent antibodies is described. Tobacco plants are examined at known intervals after infection with tobacco mosaic virus. Viral protein is demonstrable already 45 hours after infection. It appears first in the cytoplasm, in a perinuclear zone, and spreads subsequently over the entire cytoplasm. No virus protein is seen in the nuclei and chloroplasts. Synthesis of viral protein consequently seems to occur exclusively in the cytoplasm. This fact is supported also by fractionation of infected tissue. Here the fluorescent antibody reveals viral antigen principally in the microsomal fraction.

The method developed by Coons (1) for the demonstration of antigen by means of fluorescent antibodies proved eminently suited to the study of the propagation of viruses pathogenic for animals (2). We therefore tried to adapt this technique to our investigation of tobacco mosaic virus (TMV) biosynthesis. The rate at which the virus reproduces is dependent upon external conditions. Schramm and Engler (3) studied the genesis of TMV in Nicotiana tabacum at 23°C and constant illumination. The first mature virus particles were recorded by means of the local lesion test only after a latent period of 20-30 hours. This period has several causes. A certain amount of time is required to liberate ribonucleic acid (RNA) from the virus, since infection with free RNA shortens the latency by about 10 hours. Another reason for the delayed appearance of mature virus particles involves the fact that free RNA replicates before the commencement of protein synthesis. Protein combines with free RNA to form mature particles at the rate at which protein is synthesized, resulting in a decrease of free RNA after a maximum has been reached.

A thorough understanding of the manner in which TMV is formed requires knowledge of the cellular structures in which individual stages of synthesis are achieved. Shortly after infection, Zech (4) observed an increase in the absorption of nucleic acid within the cell nucleus. Thus viral RNA is probably formed in the cell nucleus. Since RNA is not antigenic, serological methods reveal little about the location of

protein synthesis. There is no positive information about this site. Electron-microscopic observations and other studies (5) suggest that chloroplasts may be involved in the synthesis of viral protein.

The present work aimed at the localization of virus protein in the tissue of Nicotiana tabacum at certain intervals after infection, based on test conditions described by Schramm and Engler (3).

Materials and methods

Preparation of labeled antiserum against TMV

For the production of antiserum, we dissolved 90-100 mg TMV in physiological saline and injected increasing dosages of this solution intravenously into a rabbit within 3-5 days. Blood was drawn 6-8 days after the last injection; serum was extracted after standing for 2 hours.

Antibodies were purified and concentrated by precipitation with an equal volume of saturated ammonium sulfate solution according to Coons' (1) instructions; the precipitate was absorbed in some phosphate buffer of pH 7.2 (1 part m/10 phosphate buffer and 4 parts physiological saline); ammonium sulfate was removed by dialysis against the same buffer lasting 3-4 days. Nitrogen was determined by Kjeldahl's method and gave the solution's protein concentration.

The fluorescein isocyanate required for coupling to antiserum is unstable and was therefore prepared freshly in each case from amino-fluorescein. Both isomers forming during preparation were used: they differ neither in their ability to react with serum nor in the intensity of fluorescence. 0.05 mg aminofluorescein are needed per mg protein.

Aminofluorescein was converted to isocyanate in a ground glass apparatus in acetone solution (under exclusion of water) by introduction of phosgene. At the end of reaction, excess acetone was evaporated in vacuo, and the gummy, brownish residue was absorbed in some dioxane-acetone mixture (1:1). Meanwhile the antiserum was diluted to 10 mg protein/cc solution according to Coons and cooled in the ice bath. Fluorescein isocyanate solution was added drop by drop with simultaneous and rapid stirring. The mixture was stirred for 18 hours more in the cold. In order to remove uncoupled stain, the solution was dialyzed for 4-6 days against phosphate buffer, pH 7.2; the buffer was renewed repeatedly. Coons recommends that the solution of fluorescent antibody be shaken after dialysis for two periods of one hour with rabbit liver powder precipitated with acetone. This prevents non-specific fluorescence.

Plant material

Plants of Nicotiana tabacum L., variety Samsun, were grown in the greenhouse. About 7-9 weeks after seeding, the plants had attained a height of 15-20 cm and had developed 3-4 leaves. At this stage they were infected with a solution of 10^{-6} g TMV/cc in phosphate buffer, pH 7.2. After dusting with carborundum, this solution was applied with a glass spatula to all mature leaves of the plant. Following infection, the leaves were rinsed with tap water and generally washed with TMV antiserum in order to inactivate TMV particles adhering to the outer surface. Finally the plants were maintained in a thermostat between 23 and 25°C under constant illumination.

Preparation of tissue sections

Fixation. Part of the tissue pieces cut from leaves (ca. 1x1 cm) were fixed. Carnoy's mixture (60 ml ethanol, 30 ml chloroform, 10 ml glacial acetic acid) is particularly suited for fixation. For comparative purposes we also used the mixtures of Bouin-Duboscq-Brasil, Stieve, Susa and Zenker, as described by Romeis (6). Fresh tissue pieces were placed for 10-20 minutes in the freshly prepared fixing solution. When fixation was too lengthy, the tissue showed pronounced shrinkage. Preparations were exposed to absolute ethyl alcohol for 15-30 minutes and subsequently processed through a decreasing alcoholic series of 90%, 70% and 40% for 2-3 minutes each, then placed in phosphate buffer of pH 7.2. The specimens were left there for 1-10 hours with frequent replacement of buffer.

Imbedding. Fixed and unfixed tissue sections are covered with gelatin solution in small aluminum dishes. The imbedding material was made by dissolving 5 g of gelatin in 20 cc distilled water and 1 cc glycerin at 37-40°C. After the gelatin had solidified in the cooler, the blocks were frozen with solid carbonic acid on a freezing microtome table and sectioned. A Leitz freezing microtome was used in a cryostat at -20 to -40°C. Sections were usually 25 microns thick. At this thickness the tissue remains coherent without the disadvantage of excessive layers of cells. The sections are placed on slides which are covered with a thin film of glycerin-gelatin for better adherence. This solution was produced by dissolving 10 g gelatin in 60 cc warm water, adding 50 cc glycerin, filtrating through gauze and addition of 1 g phenol.

After melting, the slides are stored at +4°C in a humid chamber until ready to stain.

Staining with fluorescent antibody

Fluorescent antiserum was placed on the slides until the sections were completely covered, then left in place for 45-60 minutes in a humid chamber at room temperature. Next, the serum was rinsed off briefly and the sections were washed in phosphate buffer of pH 7.2 for 10-15 minutes. The sections were then enclosed in glycerin-water under a cover glass.

The fluorescence microscope

Studies were made with a Zeiss-WL research microscope equipped with a large fluorescence apparatus and provisions for observation in the dark field, bright field and phase contrast. The fluorites (neofluars) used were designed as phase contrast objectives. A magnification adjuster (optovar) with factors 1, 1.6 and 2.5 X as well as an auxiliary lens (Amici-Bertrand lens) for the phase contrast equipment, were introduced between the tube and the tube carrier.

The large gluorescence apparatus for excitation of blue-violet and ultraviolet included a lamp with built-in high vapor pressure mercury arc OSRAM HBO 200 with an extraordinarily high luminous density of 25000 stilb at a light flux of 9500 lumen. Two BG 12 filters, each 3 mm thick, proved to be suitable excitor filters. This combination resulted in intense blue-violet response at which fluorescence was observed most favorably.

For the purpose of inclining the UV flow into the microscope, a semi-permeable mirror was attached to the base of the microscope. Observation in the bright field, phase contrast and dark field was made with the aid of a low voltage lamp incorporated in the base of the microscope. The semi-permeable mirror attached above the lamp passed its light without hindrance. Since this source eclipsed the UV lamp by far, both types of observation were readily conducted at the same time by simply switching the low-voltage lamp on and off. In order to filter out the short-waved light still extant after passage through the preparation prior to the study of fluorescence, a combination of two suppression filters for blue, OG 4 / OG 5 were installed above the optovar within an intermediate filter tube.

The most important prerequisites for achievement of ideal observation, i.e., maximal light intensity and a non-fluorescing optical system, were met in this microscope.

Exposures were made with accessories for microphotography by Zeiss. Agfa 13/10° DIN films were used for black and white, and Agfa 18/10° DIN for color.

Test results

Preliminary tests

In order to find optimal conditions for staining with fluorescent antiserum, we initiated preliminary tests with tobacco plants infected some time ago and for this reason contained a large amount of virus. We soon established that intact tissue of infected plants, e.g., peeled portions of epidermis, does not react and that labeled antibodies enter the cell only in places where the membrane is injured. Reproducible results therefore depended on the development of a suitable cutting technique. The cellular contents and structures should be changed as little as possible. The technique described in detail in the experimental part proved satisfactory. Observations with the phase contrast microscope and control stains with acridine orange showed that this cutting technique does not result in noticeable changes or losses in cellular elements upon careful application.

After treatment with fluorescent antiserum, infected tissue sections revealed a characteristic yellowish-green fluorescence under the fluorescence microscope, while untreated sections merely showed red fluorescence of chloroplasts and white-gray from vessels and calcium oxalate cells. Comparative exposures 1 and 2 merely reflect differences in the intensity and distribution of fluorescence, while color distinctions, which are quite apparent upon direct observation, naturally do not enter into play.

A number of fixing agents was found which do not impair specific yellowish-green fluorescence, e.g., those of Carnoy, Stieve and Zenker (6). Fixation with formol, on the other hand, was unsatisfactory.

The reliability and specificity of demonstration was tested in numerous control experiments. Fixed and unfixed sections were washed with phosphate buffer before exposure to antiserum. This solvent extracted TMV from unfixed sections with relative ease, whereas fixed specimens remained unaffected. Complexes which are insoluble in phasphate buffer are formed after treatment with antiserum. In addition, we examined the extent to which TMV particles may shift during cutting. Sections from non-infected tissue were treated for ½ hour with a TMV solution concentrated at 10^{-2} to 10^{-6} g/cc, then washed briefly with phosphate buffer. Very slight fluorescence, distributed diffusely over the whole tissue was seen only at the maximal TMV concentration. Thus TMV particles are not adsorbed mechanically on cellular elements; careful manipulation reveals the original viral localization.

To prove that the observed fluorescence is characteristic solely for the TMV antigen-antibody precipitate, we treated sections from infected tissue with antiserum from which specific antibodies against TMV had been removed by exhaustive precipitation. No fluorescence was observed in connection with this exhausted antiserum, while sections of

the same tissue gave strong fluorescence upon treatment with complete antiserum. Specific staining of TMV-containing tissue was not seen in the presence of a heterologous antiserum, e.g., against the virus of classical fowl cholera, nor did fluorescent antiserum against TMV react with plant tissue containing potato Y virus. As further proof of the specificity of fluorescent staining, the following control test was carried out, as recommended by Coons. We treated infected tissue sections with unlabeled TMV antiserum, thereby neutralizing virus antigen present in the cells. Subsequent treatment with fluorescent antiserum produced little or no fluorescence.

The method proved especially feasible for the examination of systemic disease in Nicotiana tabacum. Sections of local lesions produced by TMV on Nicotiana glutinosa were also studied. Occasional weak, non-specific fluorescence was observed in an irregular distribution across the necrotic tissue. Precise localization was not possible, since the necrotic cells had undergone radical changes. Since necrosis is fully developed on infected leaves 2-3 days after infection, it is likely that the cells die too rapidly and do not attain virus concentrations needed for positive fluorescence.

The occurrence of TMV protein in the course of infection

Young tobacco plants (Samsun) were maintained in the thermostat after infection with TMV under the conditions described above. 4-8 plants were used in each test. At a certain time after infection, one primarily infected leaf was cut off and 2-3 tissue pieces were procured from a certain area of the leaf. Some were fixed, others remained unfixed during treatment with fluorescent antiserum. A distinct correlation was observed between the intensity and location of fluorescence on one hand, and the duration of infection on the other. Viral protein was never seen earlier than 45 hours after infection. Initially only a fraction of the plants involved showed signs of TMV; fluorescence was weak and usually localized only at certain points. The percentage of positively reacting plants increased rapidly in the manner reflected in Fig. 1. About 100 hours after infection, all preparations showed large amounts of TMV under the fluorescence microscope. The intensity and distribution of fluorescence did not change in the subsequent course of infection. The difference between fixed and unfixed sections was slight. TMV was seen a little earlier in fixed sections, but specific fluorescence as a rule did not attain the same intensity as in unfixed sections. It is conceivable that TMV in fixed sections is more solidly bound to the point of genesis, and for this reason reaches the minimal amount necessary for observation more rapidly. At higher concentrations of TMV, on the other hand, the fixing agent probably weakens fluorescence.

Specific fluorescence appears first in primarily infected epidermis and in the vascular parenchyma. From here it spreads very quickly over the whole leaf. The localization of viral protein within individual cells was subjected to particularly thorough study. In the initial stages of infection, ca. 45-60 hours after infection, fluorescence is frequently seen only in a narrow zone around the nucleus. An example of this early stage is depicted in Fig. 3 a and b. Since not all cells are infected simultaneously, one also finds cells in which fluorescence has already spread throughout the whole cytoplasm. Exposures 4 a and b, made 48 hours after infection, are typical of this stage. It is particularly evident that the chloroplasts and nuclei invariably remain dark, even in late stages, and that no TMV protein is demonstrable in them. Characteristi examples are seen in Fig. 5-10. Fluorescence is especially clear in the plasma proximal to the wall of epidermal cells in Fig. 6, which are free of chloroplasts. In later stages of infection TMV frequently separates in crystalline form near the cell nucleus. These crystals reflect particularly strong fluorescence, as shown by Fig. 5 a and b. Only a few examples out of several hundreds of observations have been reproduced. They never revealed fluorescence in the cell nucleus or in chloroplasts. The absence of fluorescence definitely is not due to the circumstance that these cellular elements are enveloped by membranes which are impassable for fluorescent antibodies. Considering the great number of preparations, it is certain that chloroplasts and nuclei had been sliced, and that these would have taken the stain. Nor is it probable that nuclei contain fluorescence-inactivating substances, since studies of classical fowl cholera virus have shown specific fluorescence of a virus antigen precisely within the cell nucleus. It is highly probable, therefore, that cell nuclei and chloroplasts do not contain TMV protein.

Studies of cell fractions

After we had established that TMV protein occurs exclusively in the cytoplasm, the next step was to identify the components of the plasma which are responsible for protein synthesis or storage. Cells with copious virus and non-infected tissue were homogenized in 0.5-m sucrose and subjected to fractional centrifugation. This resulted in fractions listed in Table 1. Individual fractions were suspended in fluorescent antiserum, after which the antiserum was removed by centrifugation and washing with phosphate buffer. In control tests with healthy plants, the fluorescent antiserum was completely removed except for the fraction settling out at 30000 rpm. For reasons as yet unknown, antiserum is more strongly adsorbed on this fraction. Microscopically demonstrable fluorescence is very weak in more rapidly sedimenting fractions of infected tissue, including 10000 rpm. Fluorescence increased very conspicuously in fractions sedimenting at 15000 and 30000 rpm. These fractions were free of cellular debris, nuclei, chloroplasts and starch granules. Since the 30000 rpm fraction adsorbed fluorescent antiserum in the controls, nothing may be said about the TMV content of this

fraction. The strong fluorescence of the 15000 rpm fraction, on the other hand, speaks unequivocally in favor of a high TMV protein concentration. It is interesting that this fraction contains essentially microsomes, which play an important role in the synthesis of normal proteins, according to Zamecnik et al. (7). Thus fractionization tests confirmed the results of staining experiments carried out in situ. Rapidly sedimenting fractions, containing cell nuclei and chloroplasts, failed to indicate viral protein, while the microsomal fraction derived from the cytoplasm gave a clearly positive reaction.

Discussion of results

The findings reported here supplement rather favorably the studies of TMV biosynthesis described earlier. We pointed out that RNA synthesis, which takes place prior to protein synthesis, probably occurs in the cell nucleus. Numerous studies of the importance of RNA in normal protein synthesis indicate that RNA formed in the nucleus may shift to the cytoplasm in order to initiate protein synthesis there. This is apparently true also of the elaboration of viral protein, since the latter occurs initially near the nucleus and spreads subsequently to the entire cytoplasm. The microsomes are particularly active sites of normal plasmatic protein synthesis. It is interesting that a relatively high concentration of TMV protein was indicated in the microsomal fraction examined by us. We may assume, therefore, that TMV protein is also formed essentially in the microsomes. Occasional electron-microscopic findings which suggest that TMV particles accumulate on chloroplasts cannot be used as counter-arguments and probably are based on non-specific adsorption.

Fig. 1. Curve 1: Correlation between demonstration of fluorescence and time p.i.

Table 1. Fractionization tests with infected and non-infected tissue of Nicotiana tabacum. Sedimentation was made in 0.5-m sucrose, 0.02-m $MgCl_2$, 0.02-m potassium phosphate buffer, pH 7.

Rpm	Time in min.	Fraction components	Fluorescence in healthy tissue	Fluorescence in infected tissue
300	10	coarse tissue remnants and cellular debris	±	+
800	10	cellular debris and nuclei	±	+
4400	15	chloroplasts & starch granules	±	+
10000	25	broken & small chloroplasts, small starch granules	±	+
15000	30	yellowish-white sediment	±	+ + +
30000	60	gel-like, grayish-white sediment	+ + +	+ + +